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Diversity of Sulfur-Oxidizing Bacteria in Greenwater System of Coastal Aquaculture

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Abstract Reduced sulfur compounds produced by the metabolism are the one of the major problems in aquaculture. In the present study, herbivorous fishes have been cultured as biomanipulators for secretions of slime, which enhanced the production of greenwater containing beneficial bacteria. The genes encoding *soxB* which is largely unique to sulfur-oxidizing bacteria (SOB) due to its hydrolytic function has been targeted for examining the diversity of SOB in the green water system of coastal aquaculture. Novel sequences obtained based on the sequencing of metagenomic clone libraries for *soxB* genes revealed the abundance of SOB in green water system. Phylogenetic tree constructed from aligned amino acid sequences demonstrated that different clusters have only 82–93% match with *Roseobacter* sp., *Phaeobacter* sp., *Roseovarius* sp., *Sulfitobacter* sp., *Ruegeria* sp., and *Oceanibulbus* sp. The level of conservation of the *soxB* amino acid sequences ranged from 42% to 71%. 16S rRNA gene analyses of enrichment culture from green water system revealed the presence of *Pseudoxanthomonas* sp., which has 97% similarity with nutritionally fastidious Indian strain of *Pseudoxanthomonas mexicana*—a sulfur chemo-lithotrophic γ-proteobacterium. Our results illustrate the relevance of SOB in the functioning of the green water system of coastal shrimp aquaculture for oxidation of reduced sulfur compounds, which in turn maintain the sulfide concentration well within the prescribed safe levels.

Keywords Diversity · Sulfur-oxidizing bacteria · *soxB* gene · 16S rRNA · Greenwater · Coastal aquaculture

Introduction

Intensive coastal aquaculture leads to low dissolved oxygen, high-nutrient, organic, metabolic, and microbial loads. Accumulation of these metabolites causes eutrophication

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and stress among the animals. Sachidananadmurthy and Yajurvedi [1] reported higher levels of hydrogen sulfide in aquaculture body in Mysore City, Karnataka, India throughout the study period, which are due to the entry of agricultural runoff and occasional flow of sewage into the lake. There is still lack of effective means for the treatment of culture and wastewater. To maintain healthy ecosystem in aquaculture ponds, bioremediation in zero exchange and integrated recirculating systems and treatment of wastes before discharge are the best eco-friendly practices. Green water culture system is an innovative bioremediation or bioaugmentation technique through green water system, wherein economically important finfish species are also stocked as bioremeditors in the growing pond and also reservoir. In pond environment, finfish, mainly the gray mullet (*Mugil cephalus*) and milkfish (*Chanos chanos*) [2, 3], stocked with the shrimps serve as bioremediator/biomanipulator by feeding on the uneaten feed and algae, etc., and the secretions of slime from this fish enhance the production of green water which help condition the water [4, 5]. Gray mullet and milkfish are native euryhaline group of fish commonly found in estuaries and creeks. Their broad diet and tolerance to poor water quality makes them an ideal candidate for aquaculture waste treatment consumes detritus and algal growth, which in turn limit ammonication by consuming particulate organic matter and increasing the oxygen penetration to the sediments [3, 6]. However, the mechanisms on how the system works to bioremediate in zero exchange and green water recirculation system are yet to be ascertained [2, 7]. The biochemical importance of indigenous bacterial population indicates a need for development of reliable methods for identification of these organisms in nature. Molecular detection systems which do not rely on traditional cultivation appear to be promising alternatives for our current understanding of microbial populations in a wastewater system [8, 9]. Sulfur-oxidizing bacteria uses reduced inorganic sulfur compounds as growth supporting electron donors for their energy generating systems in aquatic environment. Ghosh and Rao [10, 11] have isolated sulfur chemo-lithotrophic α- and γ-proteobacteria from the rhizosphere of an Indian tropical leguminous plant and characterized them as *Mesorhizobium thiogangeticum* strain SJT^{T}, *Azospirillum* sp. TTS, and *Pseudoxanthomonas mexicana* strain JT002 based on 16S rRNA gene approach. A bacterium (*Marichromatium bheemlicum* sp. strain JA124(T)) was isolated by Kumar et al. [12] from a marine Indian aquaculture pond. Phylogenetic analysis on the basis of 16S rRNA gene sequences showed that strain JA124(T) clusters with species of the genus *Marichromatium* belonging to the class Gammaproteobacteria. A α-proteobacterium has been isolated by Srinivas et al. [13] from an Indian aquaculture pond and characterized as *Rhodovulum imhoffii* strain JA125T based on 16S rRNA gene. This has been found to utilize sulfide, sulfate, thiosulfate, and thioglycolate as sulfur sources. However, to our knowledge, nothing is known about diversity of SOB in green water system of coastal aquaculture using *soxB* functional gene approach. In phylogenetic and environmental studies, the presence of a *soxB* gene has been used as a marker for the presence of the Sox pathway and as an indicator of the ability of the organism to oxidize thiosulfate [14–18]. The aim of the present study is to detect and characterize sulfur-oxidizing bacteria in green water system of coastal aquaculture through 16S rRNA gene approach and creation of meta-genomic clone libraries of *soxB* genes.

Materials and Method

Production of Greenwater

Economically important finfish mullet—*M. cephalus* (weight 97–177 g, length 22.5 to 27.5 cm) were cultured in tank in order to produce green water, which was subsequently

analyzed for environmental physicochemical parameters and sulfur-oxidizing bacterial population.

DNA Isolation

Greenwater samples (5 L) collected from tank containing mullet was transferred to ultra-filtration unit to filter the bacteria onto the membrane (0.2 μm). The membrane filter containing bacteria was used for genomic DNA isolation using modified phenol chloroform method [19]. Genomic DNA extracted from green water was subsequently quantified using Nanodrop at 260 nm. Purity was determined by measuring the 260/280 nm absorbance ratio.

PCR Amplification of *soxB*

The following different sets of primers originally designed by Petri et al. [14] were used for the qualitative detection of sulfur-oxidizing bacteria:

soxB693F+soxB1446B (753 bp)
soxB693F+soxB1164B (488 bp)
soxB1164F+soxB1446B (282 bp)

The following primers with less degeneracy were also used:

soxB693F+soxB1446B143 (753 bp)
soxB693F+soxB1164B145 (488 bp)
soxB1164F+soxB1446B143 (282 bp)

The polymerase chain reaction was performed on the samples along with negative control (water) with a 40-μl reaction mixture using Eppendorf thermal cycler (Master cycler gradient). The following composition was used for a single reaction: (1×) 40 μl: water 27.4 μl; buffer (10 x Tris with 15 mM $MgCl_2$)**:** 4 μl, 10 mM dNTP (2.5 mM): 2 μl, forward primer (30 pM) 2 μl, reverse primer (30 pM) 2 μl, *Taq* (5U/μl) 0.2 μl, BSA (20 mg/ml) 0.4 μl, DNA template 2 μl. The amplification programs were as follows (Petri et al. [14]): 1 cycle consisting of 94°C for 2 min, followed by 10 cycles consisting of denaturation (94°C for 30 s), annealing (55°C for 40 s), and elongation (72°C for 30 s). Then, additional 25 cycles were performed with annealing temperature of 47°C and a final extension step consisting of 72°C for 6 min. The following gradient amplification program was also used: 1 cycle consisting of 94°C for 2 min, followed by 32 cycles consisting of denaturation (94°C for 30 s), annealing (50 and 54°C for 30 s), elongation (72°C for 30 s), and a final extension step consisting of 72°C for 5 min. Aliquots (8 μl) of the amplification products were electrophoresed on 1% agarose gels by using standard electrophoresis procedures.

Cloning and Sequence Analysis

The amplified *soxB* genes of different fragments 282, 488, and 753 bp were purified with a gel extraction kit (QIAquick) by following the manufacturer's instructions. The purified PCR products (282, 488, and 753 bp *soxB*) was ligated by using the pDK101 as recommended by the manufacturer and were transformed into high-efficiency competent cells. Clones were confirmed by using *NcoI* restriction endonuclease. Sequencing was done in an ABI 3100 Genetic Analyzer. Primary sequences were analyzed using the BLAST tool of www.ncbi.nlm.nih.gov.

The deduced amino acid sequences were aligned using the Clustral W program. Gaps and missing sequence information present in more than one sequence were excluded from calculation of distances and trees yielding datasets of amino acids. The phylogenetic tree based on the alignment of various fragments of *soxB* genes were generated by using the maximum likelihood method from PHYLIP 3.57 program package. Bootstrap analyses of nucleotide and amino acid sequences were performed with the program.

Isolation and Characterization of Strain

From green water system, a chemolithotrophic bacterial strain DBTS3 has been retrieved by enrichment using MS–thiosulfate–yeast extract as proscribed by earlier researchers [10, 11]. Genomic DNA was isolated from the isolate, and near-complete 16S rRNA gene of the strain was amplified using universal primers, sequenced and phylogenetically analyzed.

Results and Discussion

Greenwater Quality Parameters

Analysis of greenwater revealed that water quality parameters such as salinity (20±1 ppt), pH (7.7–8.1), nitrite–N (0.05–0.07 mg/l), total ammonia N (0.12–0.28 mg/l), phosphates–P (0.06–0.12 mg/l), chemical oxygen demand (68–76 mg/l), biochemical oxygen demand (6–10 mg/l), and sulfide (Bdl to 0.002 mg/l) were found to be well within the safe levels.

Detection of Sulfur-Oxidizing Bacteria in Greenwater

Sulfur-oxidizing bacteria have been detected in green water system using PCR amplification of s*oxB* genes. As shown in Table 1, different set of primers originally designed by Petri et al. [14] were used for the qualitative detection of sulfur oxidizing bacteria. Primers with less degeneracy were also used. Different combinations of primers used and expected size of PCR product in the present study are listed in Table 2. Primer sets such as soxB693F+soxB1446B, soxB693F+soxB1164B, and soxB1164F+soxB1446B used in this study offered reliable amplification results and yielded PCR products of the expected length of 753 bp (Fig. 1), 488 bp (Fig. 2), and 282 bp (Fig. 3).

The original reverse primer (soxB1164B) devised by Petri et al. [14] contained a degeneracy at five positions, an R at the third and 15th nucleotides (R = A/G) and N at sixth, ninth, and 12th nucleotide (N=A/C/G/T) in the primer sequence. Another reverse primer (soxB1446B) developed by Petri et al. [14] contained degeneracy at four positions—

Table 1 Primers used for PCR amplification of *soxB* gene in the present study.

Primer	Sequence of primer	Literature
soxB693F	5′-ATCGGNCARGCNTTYCCNTA-3′	Petri et al. [14]
soxB1164F	5′-TAYCGNCGNGGNAAYTT-3′	Petri et al. [14]
soxB1446B	5′-CATGTCNCCNCCRTGYTG-3′	Petri et al. [14]
soxB1164B	5′-AARTTNCCNCGNCGRTA-3′	Petri et al. [14]
soxB1446BK143	5′-CATGTCSCCDCCBTGYTGRTA-3′	Present study
soxB1164BK145	5′-AAGTTGCCDCGNCGRTA-3′	Present study

Table 2 Expected size of PCR amplified products using specific primers.

Primer combination	Expected size of PCR product (bp)
soxB693F + soxB1446B	753
soxB693F + soxB1164B	488
soxB1164F + soxB1446B	282
soxB693F + soxB1446B143	753
soxB693F + soxB1164B145	488
soxB1164F + soxB1446B143	282

an N at the seventh and 10th nucleotides, R at the 13th nucleotide, and Y (C/T) at 16th nucleotide in the primer sequence. In this study, reverse primer—soxB1164BK145 with less degeneracy—a D (D=A/G/T) at the 10th, an N at 13th nucleotide, and an R at 16th nucleotide and another reverse primer soxB1446BK143 with less degeneracy—an S (C/G) at the 7th, D at 10th, B (C/G/T) at 13th, Y at 16th, and R at 19th were also used. Primers with less degeneracy used in the present study amplified templates with almost the same efficiency (Figs. 1, 2, 3).

Diversity of Sulfur-Oxidizing Bacteria in Greenwater System

Diversity of the *soxB* gene was examined in greenwater coastal aquaculture system though creation of metagenomic clone libraries of *soxB* genes (753, 488, 282 bp). The nucleotide sequences of *soxB* genes determined in this study have been deposited in the GenBank

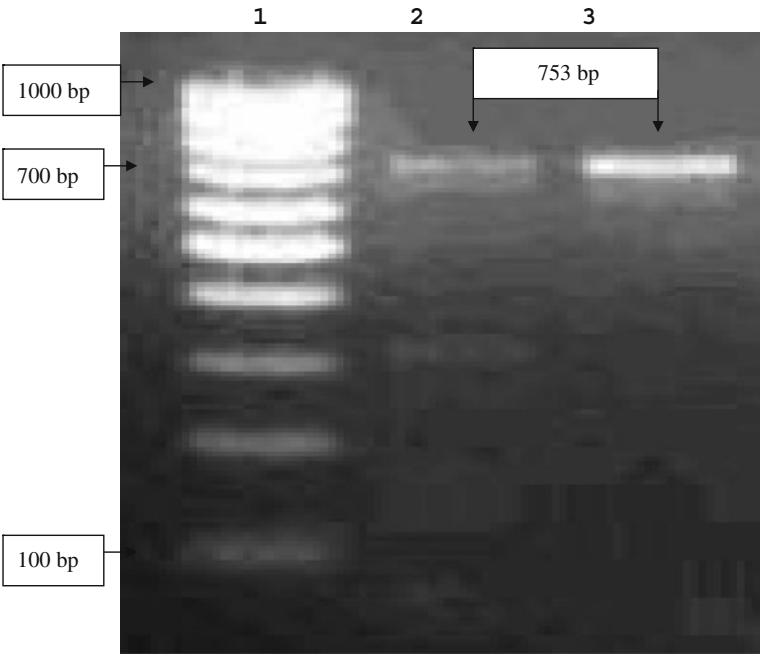

Fig. 1 PCR amplification of 753 bp (*Lane 1* 100 bp Marker, *lane 2* soxB693F+soxB1446B primers, *lane 3* soxB693F+soxB1446B143 primers)

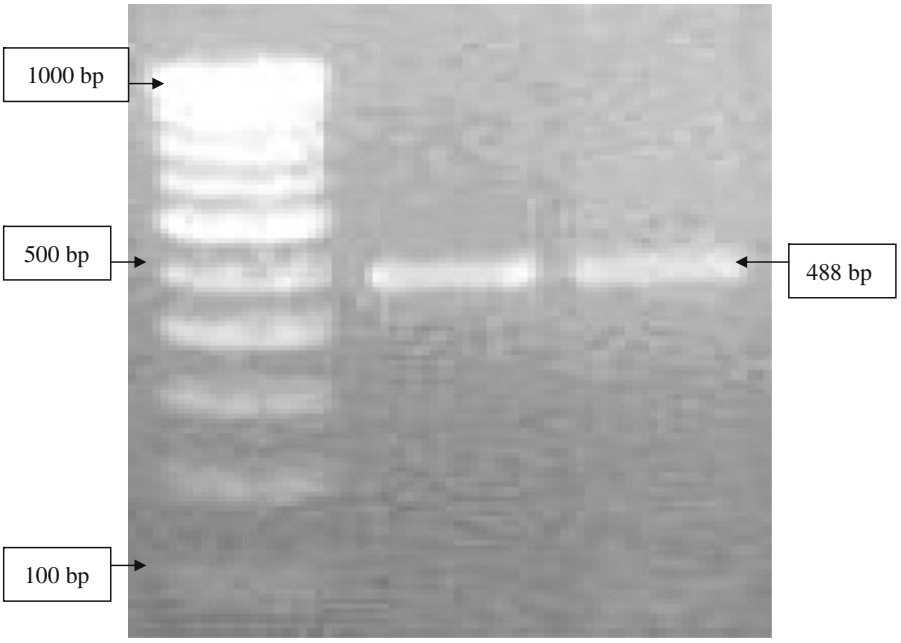

Fig. 2 PCR amplification of 488 bp fragments of *soxB* gene (*Lane 1* 100 bp Marker, *lane 2* soxB693F+ soxB1164B primers, *lane 3* soxB693F+soxB1164B145 primers)

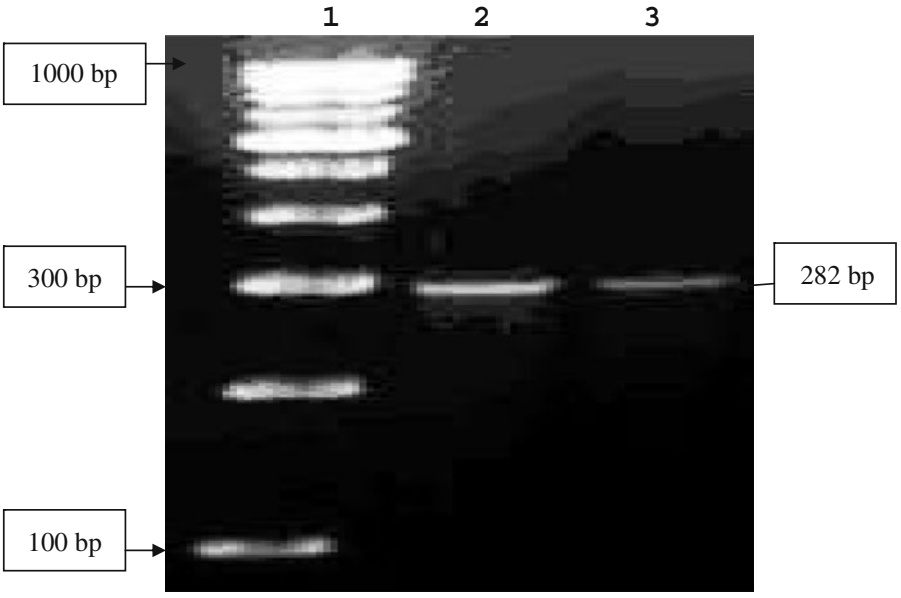

Fig. 3 PCR amplification of 282 bp fragment of the *soxB* gene (*Lane 1* 100 bp Marker, *lane 2* soxB1164F+ soxB1446B primers, *lane 3* soxB1164F + soxB1446B143 primers)

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EAQ11431        IGQAFPYMPIANPGWMFPEYSFGIRDERMQEMVDEVRAAGAELVVCLSHNGFDVDKKMAG 60
EDM69506        IGQAFPYMPIANPGWMFPDYSFGIRDEHMQQMVDEVRAEGAELVVCLSHNGFDVDKKMAG 60
EDM30351        IGQAFPYMPIANPGWMFPEYSFGIRDENMQAMVDEVRGQGAELVVCLSHNGFDVDKKMAS 60
EAQ24473        IGQAFPYMPIANPGWMFPEYSFGIRDENMQAMVDEVRGQGAELVVVLSHNGFDVDKKMAS 60
EDQ07168        IGQAFPYMPIANPGWMFPEYSFGIRDEHMQDMVDEVRAAGADVVVVLSHNGFDVDKKMAG 60
EDQ14340        IGQAFPYMPIANPGWMFPEYSFGIRDEHMQDMVDEVRAAGADVVVVLSHNGFDVDKKMAG 60
EEB69750        IGQAFPYMPIANPGWMFPEYSFGIRDEHMQDMVDEVRAAGADVVVVLSHNGFDVDKKMAG 60
ACM66875        IGQAFPYMPIANPGWMFPEYSFGIRDEHIQDMVDEVRAAGADVVVVLSHNGFDVDKKMAG 60
CAC82471        IGQAFPYMPIANPGWMFPEYSFGIRDEHMQEMVDEVRAAGADVVVCLSHNGFDVDKKMAS 60
EAQ45868        IGQAFPYMPIANPGWMFPEYSFGIRDEHMQDMVDEVRAAGADLVVLLSHNGFDVDKKMAS 60
EDZ45611        IGQAFPYMPIANPGWMFPEYSFGIRDEHMQAMVDEVRATGADVVVVLSHNGFDVDKKMAS 60
EAU43863        IGQAFPYMPIANPRWMFPEYSFGIRDEHMQEMVDEVRGMGAELVVCLSHNGFDVDKKMAG 60
EAP75534        IGQAFPYMPIANPGWMFPEYSFGIRDEHMQQMVDEVRAEGAELVVCLSHNGFDVDKKMAG 60
ACM66878        IGQAFPYMPIANPGWMFPEYSFGIRDQRMQEMVDEVRAMGADWVVVLSHNGLDVDKQMAS 60
ACM66872        IGQAFPYMPIANPGWMFPEYSFGIRDQRMQEMVDEVRAMGADLVVVLSHNGFDVDKQMAS 60
ACM66876        IGQAFPYMPIANPGWMFPEYSFGIRDERMQEMVDEVRDMGAEVVVVLSHYGFDVDKKMAS 60
ACM66874        IGQAFPYMPIANPGWMFPEYSFGIRDERMQEMVDEVRDMGAEVVVVLSHNGFDVDKKMAS 60
ACM66871        IGQAFPYMPIANPGWMFPEYSFGIRDERMQEMVDEVRDMGAEVVVVLSHNGFDVDKKMAS 60
ACM66877        IGQAFPYMPIANPGWMFPEYSFGIRDERMQEMVDEVRDMGAEVVVVLSHNGFDVDKKMAS 60
ACM66873        IGQAFPYMPIANPGWMFPEYSFGIRDGRMQEMVDEVRDMGAEVVVVLSHNGFDVDKKMAS 60
CAC82472        IGQAFPYMPIANPGWMFPEYSFGIRDHRMQEVVDEVRAAGAELVVCLSHNGFDVDKQMAS 60
EDQ04241        IGQAFPYMPIANPGWMFPEYSFGIRDEHMQEMVDEVRAKGAELVVCLSHNGFDVDKKMAG 60
                ************* **** :******* .:* :*****  **: ** *** *:****:**.

EAQ11431        RVQGIDVILSGHTHDALPEPVVVGDTIIIASGSNGKFVSRVDLDVRDGQMMGFRHKLIPI 120
EDM69506        VVSGIDVILSGHTHDALPEPVLVGDTIIIASGSNGKFVSRVDLDVRDGQMMGFRHKLIPI 120
EDM30351        RVTGIDVILSGHTHDALPEPVLVGETIIVASGSNGKFVSRVDLDVRDGRMMGFRHKLIPI 120
EAQ24473        RVTGIDVILSGHTHDALPEPALVGETIIVASGSNGKFVSRVDLDVRDGRMMGFRHKLIPI 120
EDQ07168        VVSGIDVILSGHTHDALPEPVLVGQTHIVASGSNGKFVSRVDLDVRDSQLMGLRHKLIPV 120
EDQ14340        VVSGIDVILSGHTHDALPEPVLVGQTHIVASGSNGKFVSRVDLDVRDSQLMGLRHKLIPV 120
EEB69750        VVSGIDVILSGHTHDALPEPVLVGKTHIIASGSNGKFVSRVDLDVRDSQLRGLRHKLIPV 120
ACM66875        VVSGIDVILSGHTHDALPEPVLVGKTHIIASGSNGKFVSRVDLDVRDSQLMGLRHKLIPV 120
CAC82471        VVTGIDVILSGHTHDALPEPVLVGTTHIIASGSNGKFVSRVDLDVRDGQMMGLKHKLIPI 120
EAQ45868        VVTGIDVILSGHTHDALPEPVLVGKTHIIASGSNGKFVSRIDLDVRDGQMMGIRHKLIPI 120
EDZ45611        RVQGIDVILSGHTHDALPEPVLAGKTHIIASGSNGKFVSRVDLDVRDGQLMGLRHKLIPV 120
EAU43863        IVNGIDVILSGHTHDALPEPVLVGETIIVASGSNGKFVSRVDLDVRDGRMMGFRHKLIPI 120
EAP75534        IVTGIDVILSGHTHDALPEPVQVGDTIIVASGSNGKFVSRVDLDVRDGRMMGFRHKLIPI 120
ACM66878        RVSGIDVILSGHTHDALPEPVQVGQTFIIASGSNGKFVSRVDLDVRDGRMMGLRHKLIPI 120
ACM66872        RVSGIDVILSGHTHDALPEPVQVGQTFIIASGSNGKFVSRVDLDVRDGRMMGLRHKLIPI 120
ACM66876        RVSGIDVFLSGHTHDALPEPVLVNETIIVASGSNGKFVSRVDLDVRDGRMMGFKHKLIPI 120
ACM66874        RVSGIDVILSGHTHDALPEPVLVNETIIVASGSNGKFVSRVDLDVRDGRMMGFKHKLIPI 120
ACM66871        RVSGIDVILSGHTHDALPEPVLVNETIIVASGSNGKFVSRVDLDVRDGRMMGFKHKLIPI 120
ACM66877        RVSGIDVILSGHTHDALPEPVLVNETIIVASGSNGKFVSRVDLDVRDGRMMGFKHKLIPI 120
ACM66873        RVSGIDVILSGHTHDALPEPVLVNETIIVASGSNGKFVSRVDLDVRDGRMMGFKHKLIPI 120
CAC82472        RVTGIDVILTGHTHDALPEPVLVGETILIASGSNGKFVSRVDLDVRDGRMMGFRHKLIPI 120
EDQ04241        IVKGIDVILSGHTHDALPEPVLIGETHIIASGSNGKFVSRVDLDVRDGKMMGIRHKLIPI 120
                 * ****:*:**********.  . * ::***********:******.:: *::*****:

EAQ11431        FSDVIEADAEMTALIDEQRAPYLGELEEVIGTTDTELYRRGNFNGSWDDLICDALISERE 180
EDM69506        FSDVIEPDAEMAALIDEQRAPYQADLDEVIGQTDTLLYRRGNFNGSWDDLICEALLSERD 180
EDM30351        FSDVIEPDKEVATLIEEQRAPYLDQLTEVIGKTDSLLYRRGNFNGTWDDLICDALLSERE 180
EAQ24473        FSDVIEPDKEVAALIDAERAPFKADLEEVIGKTDSLLYRRGNFNGTWDDLICDALISERE 180
EDQ07168        FSDVIAPDPVITQLIDDQRAPYEADLAEVIGQTDELLYRRGNFNGTWDDLICDALLSERE 180
EDQ14340        FSDVIAPDPVITQLIDDQRAPYEADLAEVIGQTDELLYRRGNFNGTWDDLICDALLSERE 180
EEB69750        FADVIAPDPVISKLIDEQRAPYEAELAEVIGQTDELLYRRGNFNGTWDDLICDALLSERE 180
ACM66875        FADVIAPDPVISKLIDEQRAPYEAELAEVIGQTDELLYRRGNFNGTWDDLICDALLSERE 180
CAC82471        FSDVIAPDPVISKLIDEQRAPFKAELEEVIGQTDSLLYRRGNFNGTWDDLICDALLSERE 180
EAQ45868        FSDIIAPDPVITKLIDEQRAPYADQLAEVIGKTESLLYRRGNFNGTWDDLICDALLSERE 180
EDZ45611        FSDVIAPDPAVAQLIEAERAPFKAQLEEVIGQTDTLLYRRGNFNGTWDDLICDALLSERE 180
EAU43863        FSDVITPDAEVAKLIDEQRAPYKAELEEVIGQTDELLYRRGNFNGSWDDLICDALMSERE 180
EAP75534        FSDVITPDAEIAALIDEQRAPHAEALAEVIGRTDGLLYRRGNFNGSWDDLICDALLTRRE 180
ACM66878        FSDVITPDAAITRLIDEQRAPYEADLTKVNGQADGLLYRRGNFNGSWDDLICQALIEERE 180
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Fig. 4 Partial alignment of the predicted amino acids encoded by *soxB* gene

database. The accession numbers for the gene sequences are FJ620641 to FJ620659. The level of similarity between different pairs of *soxB* genes at nucleotide and amino acids levels ranged from 68% to 99% and 80% to 100%, respectively. This revealed that sequence difference has been resulted from actual diversity in the sample, and there was no PCR artifact.

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ACM66872        FSDVITPDAAITRLIDEQRAPYEADLTKVIGQADGLLYRRGNFNGSWDDLICQALIEERE 180
ACM66876        FSDVITPDAQVAALIDEQRAPYINELTEVIGQSESLLYRRGNFNGSWDDLICQALIEERE 180
ACM66874        FSDVITPDAQVAALIDEQRAPYINELTEVIGQSESLLYRRGNFNGSWDDLTCQALIEERE 180
ACM66871        FSDVITPDAQVAALIDEQRAPYINELTEVIGQSESLLYRRGNFNGSWDDLICQALIEERE 180
ACM66877        FSDVITPDAQVAALIDEQRAPYINELTEVIGQSESLLYRRGNFNGSWDDLICQALIEERE 180
ACM66873        FSDVITPDAQVAALIDEQRAPYINELTEVIGQSESLLYRRGNFNGSWDDLICEALIEERE 180
CAC82472        FSDVITPDAEVAAVIDEQRAPFMDQLTEVIGQADSLLYRRGNFNGTWDDLICDALMTERE 180
EDQ04241        FSDLITPDAEITKLIDAQRAPYLDDLTHVVGKSDALLYRRGNFNGTWDDLICDALLSERD 180
                *:*:* .*  :: :*: :***.   * .* * ::  *********:**** *:**: .*:

EAQ11431        ADIAMSPGVRWGPSLLPGDNITREDIWNVTSMTYPNAYRTEMTGEFIHTILEDVADNLFN 240
EDM69506        AEIAMSPGVRWGPSLIPGDNITREDIWNVTSMTYGKAYRSEMTGEFIKVILEDVGDNIFN 240
EDM30351        ADIAMSPGVRWGPSLIPGQDITREDIFNVTSMTYGKAYRSEMTGEFIKVILEDVADNIFN 240
EAQ24473        ADIAMSPGVRWGPSLIPGQDITREDLFNVTSMTYGKAYRSEMTGEFIKVILEDVADNIFN 240
EDQ07168        ADIALSPGVRWGPSLLPGDDITREDIWNVTSMSYGAAYRNEMTGEFLKVVMEDVADNIFN 240
EDQ14340        ADIALSPGVRWGPSLLPGDDITREDIWNVTSMSYGAAYRNEMTGEFLKVVMEDVADNIFN 240
EEB69750        ADIALSPGVRWGPSLLPGDDITREDIWNVTSMSYGAAYRNEMTGEFLKVVMEDVADNIFN 240
ACM66875        ADIALSPGVRWGPSLIPGDDITREDIWNVTSMTYGKAYRTEMTGEFIKVILEDVADNIFN 240
CAC82471        ADIAMSPGVRWGPSLVPGDDITREDIWNVTSMSYGAAYRSEMTGEFIKVILEDVADNIFN 240
EAQ45868        ADIAMSPGVRWGPSLVPGDDITREDIWNVTSMSYGAAYRSEMTGEFIKVILEDVADNIFN 240
EDZ45611        ADIAMSPGVRWGPSLVPGDEITREDIWNVTSMSYGEAYRSEMTGEFIKVILEDVADNIFN 240
EAU43863        ADIAMSPGVRWGPSLIPGDPITREDIWNVTSMSYGEAYRSEMTGEFIKVILEDVADNIFN 240
EAP75534        AEIALSPGVRWGPSLIPGQDITREDIWGVTSMTYPEAYRSEMTGEFLKIVLEDVADNIFN 240
ACM66878        ADISMSPGVRWGPSILPGQDITRDDIWNVTSMTYPNAYRTEMTGEFIHVIMEDVADNLFN 240
ACM66872        ADISMSPGVRWGPSILPGQDITREDIWNVTSMTYPNAYRTEMTGEFIHVIMEDVADNLFN 240
ACM66876        ADISMSPGVRWGPSILPGQDITREDIWNVTSMTYGAAYRTEMTGEFIHVILEDVADNLFN 240
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ACM66877        ADISMSPGVRWGPSILPGQDITREDIWNVTSMTYGAAYRTEMTGEFIHVILEDVADNLFN 240
ACM66873        ADISMSPGVRWGPSILPGQDITREDIWNVTSMTYGAAYRTEMTGEFIHVILEDVADNLFN 240
CAC82472        ADIAMSPGVRWGPSILPGQDITREDIHNVTSMTYGQAYRTEMTGEFLNVILEDVGDNLFN 240
EDQ04241        AEIALSPGVRWGPSILPGQDITREDIFNVTSMTYPNAYRTEMTGEFLKVVLEDVGDNLFN 240
                *:*::*********::**: ***:*: .****:*  ***.******:: ::***.*.:**

EAQ11431         PDPYYQQGGDM 251
EDM69506         PDPYYQQGGDM 251
EDM30351         PDPYYQQGGDM 251
EAQ24473         PDPYYQQGGDM 251
EDQ07168         PDPYYQQGGDM 251
EDQ14340         PDPYYQQGGDM 251
EEB69750         PDPYYQQGGDM 251
ACM66875         PDPYYQHGGDM 251
CAC82471         PDPYYQXGGDM 251
EAQ45868         PDPYYQQGGDM 251
EDZ45611         PDPYYQQGGDM 251
EAU43863         PDPYYQQGGDM 251
EAP75534         PDPYYQQGGDM 251
ACM66878         VDPYYQHGGDM 251
ACM66872         VDPYYQHGGDM 251
ACM66876         VDPYYQHGGDM 251
ACM66874         VDPYYQHGGDM 251
ACM66871         VDPYYQHGGDM 251
ACM66877         TDPYHQHGGDM 251
ACM66873         VDPYYIHGRDM 251
CAC82472         PDPYYQQGGDM 251
EDQ04241         PDPYYQQGGDM 251
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Fig. 4 (continued)

For the *soxB* genes clonal libraries (282, 488, 753 bp), nucleotides and predicted amino acids sequences of the *soxB* genes identified in this study are compared with the sequences available in the GenBank. An alignment of the amino acids in parts of the *soxB*-encoded proteins is presented in Fig. 4. Residues conserved in SoxB sequences are highlighted.

Higher bootstrap values indicated higher topological values. The deduced amino acid sequence of the novel ORF *soxB* comprised of 94, 162, and 251 amino acids residues, and a total of 40, 116, 155 amino acid residues were conserved in the clones obtained from green water system of coastal aquaculture. The level of conservation of the *soxB* amino acid sequences ranged from 42% to 71%. Among the closely related sequences, there is a high percentage of sequence similarity but significantly lower similarity values between the distantly related sequences. The average values between *soxB* sequences of alphaproteobacteria environmental clones were 82–100% for amino acid and 72–99% nucleotide sequences. The average values between the distantly related branches of proteobacteria were 80–83% for amino acid and 68–74% for nucleotide sequences.

Phylogenetic Analyses of *soxB*

Phylogenetic tree was constructed from aligned amino acid sequences to derive branching orders as shown in Fig. 5. Sixty amino acid sequences (starting with IGQAFPY) were used for tree construction. The *soxB* amino acid and nucleotide alignments yielded nearly identical trees (nucleotide data not shown here). The numbers at the nodes are the percentage of bootstrap trees within similar topologies. The phylogenetic tree indicated the abundance of the sulfur oxidizing bacterial community in green water system of coastal aquaculture. Followings environmental clones obtained from green water system have only 80–93% homology (Table 3) with *Roseobacter* sp., *Phaeobacter* sp., *Roseovarius* sp.,

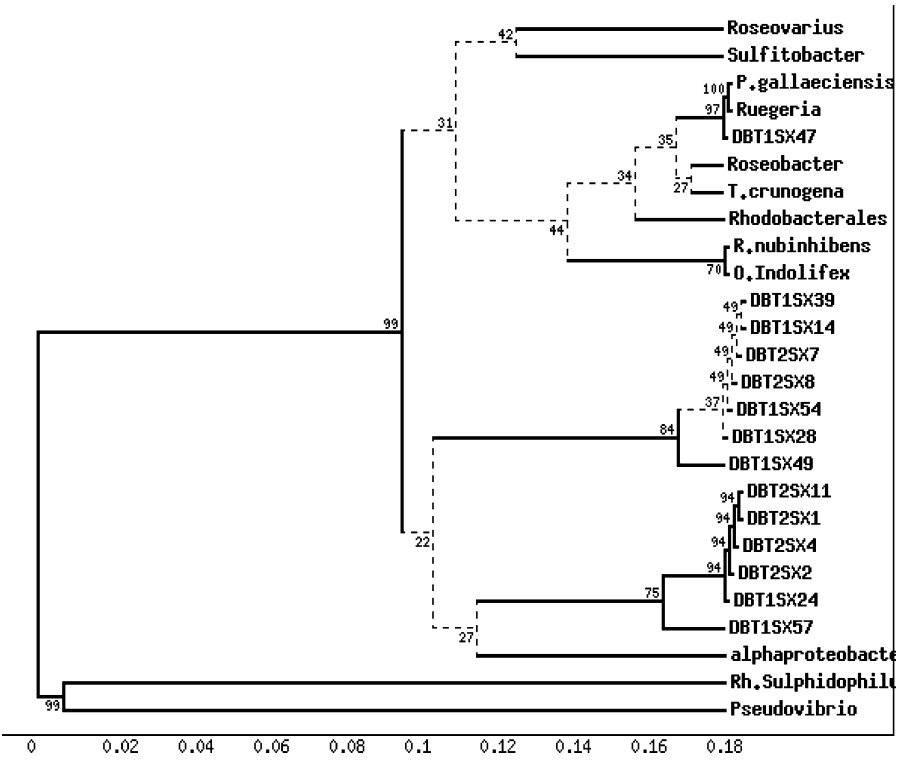

Fig. 5 Phylogenetic tree based on predicted amino acids encoded by *soxB* as determined by maximum likelihood method

Table 3 Percent similarity of amino acids predicted from 753 bp sequence to *soxB* amino acids in GenBank database.

Environmental clone	Accession number	Homology on amino acid levels	
DBT3SX25	(FJ620641-ACM66860)	*Thiocystis violacea* DSM 207 (ABR67343)	80%
DBT3SX29	(FJ620642-ACM66861)	*Roseobacter sp.* GAI101 (EEB85324)	89%
DBT3SX33	(FJ620643-ACM66862)	*Roseobacter litoralis* Och 149 (ZP_02143384)	88%
DBT3SX34	(FJ620644-ACM66863)	*Roseovarius* sp. TM1035 (EDM30351)	87%
DBT3SX36	(FJ620645-ACM66864)	*Roseobacter* sp.MED193 (EAQ45868)	93%
DBT2SX1	(FJ620646-ACM66865)	*Roseovarius* sp. HTCC2601 (EAU43863)	83%
DBT2SX2	(FJ620647-ACM66866)	alpha proteobacterium HY-103 (CAC82472)	85%
DBT2SX4	(FJ620648-ACM66867)	*Phaeobacter gallaeciensis* 2.10 (EDQ07168)	85%
DBT2SX7	(FJ620649-ACM66868)	*Roseovarius* sp. 217 (EAQ24473)	85%
DBT2SX8	(FJ620650-ACM66869)	*Roseobacter* sp. AzwK-3b (EDM69506)	85 %
DBT2SX11	(FJ620651-ACM66870)	*Phaeobacter gallaeciensis* BS107 (EDQ14340)	85%
DBT1SX14	(FJ620652-ACM66871)	*Roseobacter litoralis* Och 149 (EDQ15116)	84%
DBT1SX24	(FJ620653-ACM66872)	*Rhodobacterales* bacterium HTCC2654 (EAQ11431)	85%
DBT1SX28	(FJ620654-ACM66873)	*Sulfitobacter* sp. NAS-14.1 (EAP80119)	83%
DBT1SX39	(FJ620655-ACM66874)	*Roseobacter denitrificans* OCh 114 (ABG31148)	83%
DBT1SX47	(FJ620656-ACM66875)	*Ruegeria* sp. R11 (EEB69750)	83%
DBT1SX49	(FJ620657-ACM66876)	*Roseobacter* sp. GAI101 (EEB85324)	83%
DBT1SX54	(FJ620658-ACM66877)	*Sulfitobacter* sp. EE-36 (EAP83240)	84%
DBT1SX57	(FJ620659-ACM66878)	*Oceanibulbus indolifex* HEL-45 (EDQ04241)	82%

Sulfitobacter sp., *Ruegeria* sp., *Oceanibulbus* sp., and *Thiocystis* sp.: DBT3SX25 (FJ620641-ACM66860), DBT3SX29 (FJ620642-ACM66861), DBT3SX33 (FJ620643-ACM66862), DBT3SX34(FJ620644-ACM66863), DBT3SX36 (FJ620645-ACM66864), DBT2SX1 (FJ620646-ACM66865), DBT2SX2 (FJ620647-ACM66866), DBT2SX4 (FJ620648-ACM66867), DBT2SX7 (FJ620649-ACM66868), DBT2SX8 (FJ620650-ACM66869), DBT2SX11 (FJ620651-ACM66870), DBT1SX47 (FJ620656-ACM66875), (2)DBT1SX14 (FJ620652-ACM66871), DBT1SX24 (FJ620653-ACM66872), DBT1SX28 (FJ620654-ACM66873), DBT1SX39 (FJ620655-ACM66874) DBT1SX54 (FJ620658-ACM66877), and DBT1SX57 (FJ620659-ACM66878).

Petri et al. [14] proved the presence of *soxB*-encoding genes in eight thiosulfate-utilizing reference strains from the α, γ, and β-proteobacteria implicated in "Paracoccus sulfur oxidation" (or PSO) pathway, which involve the *soxB* gene. The α-proteobacteria converts thiosulfate to sulfate without sulfur globule formation as free intermediate. Petri et al. [14] reported that some of the thiosulfate oxidizers (*Thiomicrospira* sp.) did not yield *soxB* amplification products. This has been attributed to S4I pathway, which is enymatically different from the PSO pathway and does not involve the *soxB* gene [20, 21]. Meyer et al. [15], who studied the distribution and phylogenetic relationship of *soxB* genes among sulfur-oxidizing bacteria and detected the *soxB* gene in all investigated photo- and chemotrophic species that form sulfur globules during thiosulfate oxidation.

Ghosh et al. [22] phylogenetically analyzed SoxXA, SoxYZ, and SoxCD sequences and correlated the results with earlier SoxB-based data and postulated that the system evolved through extensive horizontal gene transfer, and all their proteins have similar evolutionary paths. Lahiri et al. [23] reported the involvement of gene cluster soxSRT-

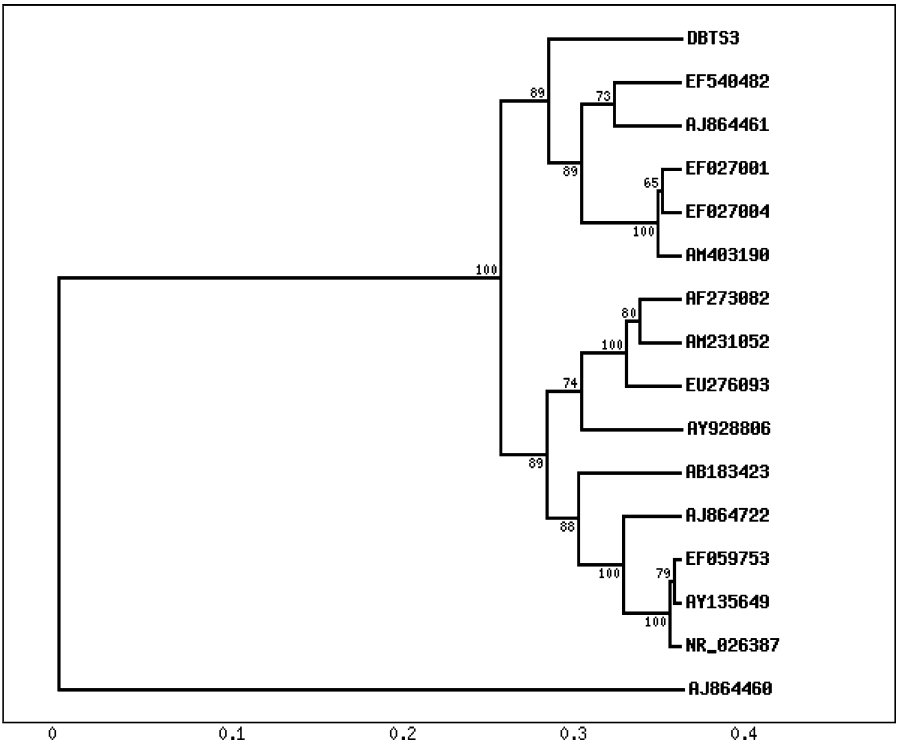

Fig. 6 Phylogenetic tree based on 16S rRNA gene sequences, showing the phylogenetic relationships of *Pseudoxanthomonas* sp. DBTS3 and related genera

soxVWXYZABCD present in the alpha-proteobacterium *Pseudaminobacter salicylatoxidans* KCT001 for chemolithotrophic oxidation of both thiosulfate and tetrathionate. While several components of their Sox systems are quite similar, i.e., the proteins SoxXA, SoxYZ, and SoxB, they differ by Sox (CD) [2] which is absent in sulfur globule-forming organisms [24]. SoxB sequences obtained in the present study exhibited only 64–66% and 55–68% homology with *P. salicylatoxidans* KCT001 on amino acids and nucleotide levels, respectively.

Wodara et al. [25] have reported identification and sequence analysis of *soxB* gene of α-proteobacterium (*Paracoccus denitrificans GB17*), which is a Gram-negative neutrophilic, facultatively chemolithoautotrophic eubacterium that oxidize thiosulfate and sulfide to sulfate and grows heterotrophically with a large variety of carbon sources. The function of the *soxB* gene product of *P. denitrificans GB17* was found to identical with that of enzyme B of the thiosulfate-oxidizing enzyme system of *T. versutus* [26]. SoxB sequences submitted in the GenBank exhibited only 69–75% and 72–79% homology with *P. denitrificans GB17* on nucleotide and amino acids levels, respectively.

Roseobacter sp. strain MED193 is closely related to *Roseobacter denitrificans*. The ability of *Roseobacter* clade members to metabolize sulfur compounds has been well studied [27], whereas *R. denitrificans* has not been studied in the same thorough manner [28]. Genome codes of *R. denitrificans* for many of the same conserved sulfur metabolic pathways revealed the further mechanism for energy metabolism. Along with phototrophy and denitrification, inorganic sulfur oxidation, encoded by the large *soxWXYZABCDEF*

cluster, is a likely source for extra energy production yielding sulfate as environmental output. Environmental clones obtained in the present study have only 79–84% homology with *R. denitrificans* strain OCh 114 on amino acids levels.

Description of *Pseudoxanthomonas* sp. DBTS3

Green water system of coastal aquaculture found to be rich in phylogenetic and metabolic diversity of sulfur chemolithotrophic bacteria. One such phylogenetically novel isolate *Pseudoxanthomonas* sp. strain DBTS3 has been isolated. The GenBank accession number for the 16S rRNA gene is GU122958. Comparison with sequences available in NCBI database identified DBTS3 as a member of gamma subclass of Proteobacteria. 16S rRNA gene sequence-based phylogentically analysis revealed that isolate had 98% similarity to *P. mexicana* and *Pseudoxanthomonas japonensis* and occupied the same phylogenetic branch as *Pseudoxanthomonas* species (Fig. 5). Species of other genera, i.e., *Pseudomonas boreopolis*, *Stenotrophomonas* exhibited 96% homology. There was 95% 16S rRNA gene similarity with the species of *Xanthomonas*, i.e., *Xanthomonas campestris*, *Xanthomonas cucurbitae*, and *Xanthomonas axonopodis.* This isolate exhibits 97% homology with *P. mexicana* strain JT002 isolated by Ghosh and Roy [11] from the rhizosphere of an Indian trophical leguminous plant, whereas, *M. thiogangeticum* and *Azospirillum* sp. isolated by Ghosh and Rao [10] had lower 16S rRNA gene sequence similarity (77%) to DBTS3. Molecular investigation of sulfur chemolithotrophy in bacteria like the ones described here is likely to help understand the evolution, diversification, and spread of this unique metabolism (Fig. 6).

The present study based on the examination of the diversity of sulfur-oxidizing bacteria through creation of metagenomic clone libraries for *soxB* genes helps in understanding of different clusters of sulfur oxidizing bacteria in green water system of coastal aquaculture, which maintain the sulfide concentration well within the prescribed safe levels. 16S rRNA gene analyses revealed the presence of *Pseudoxanthomonas* sp. a chemolithotrophic γ-proteobacterium. Presence of sulfur-oxidizing bacterial clusters in green water system may have been evolved through extensive horizontal gene transfer. Successful further studies on the involvement of sox gene cluster present in isolate will be beneficial to ascertain spread of sulfur oxidation metabolism.

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References

1. Sachidanandamurthy, K. L., & Yajurvedi, H. N. (2006). A study on physicochemical parameters of an aquaculture body in Mysore city, Karnataka. *India J Environ Biol, 27*(4), 615–618.
2. Baliao, D. D., De Los Santo, M. A., & Franco, N. M. (1999). *Milkfish pond culture. Aquaculture Extension manual no. 25*. Ilo-ilo: SEAFDEC.
3. Baliao, D. D. (2000). *Environment-friendly schemes in intensive shrimp farming. State of the art Series*. Ilo-ilo: SEAFDEC.
4. Bagarinao, T. (1998). Historical and current trends in milkfish farming in the Philippines. In S. S. de Silva (Ed.), *Tropical mariculture* (pp. 381–422). London: Academic.
5. de la Rosa, J. S. (2004). *Greenwater technology: A new shrimp culture technique*. BAR Digest, http://www.bar.gov.ph/bardigest/2004/janmar04_greenwater.asp
6. Yearlsley, G. K. (1999). *Australian seafood handbook*. Hobart: CSIRO Marine Research.

7. Tookwinas, S. (2000). *Closed-recirculating shrimp farming system. State of the art series*. Ilo-ilo: SEAFDEC.
8. Krishnani, K. K., Shekhar, M. S., Gupta, B. P., & Gopikrishna, G. (2009). Sequence similarity based identification of nitrifying bacteria in coastal aquaculture for bioremediation predictability. *Asian Fisheries Science, 22*, 41–49.
9. Krishnani, K. K., Shekhar, M. S., Gopikrishna, G., & Gupta, B. P. (2009). Molecular biological characterization and biostimulation of ammonia oxidizing bacteria in brackishwater aquaculture. *Journal of Environmental Science and Health, A-l44*(14), 1–11.
10. Ghosh, W., & Roy, P. (2007). Chemolithotrophic oxidation of thiosulphate and tetrathionate by novel strains of *Azospirillum* and *Pseudoxanthomonas* isolated from the rhizosphere of an Indian tropical leguminous plant. *Current Science, 93*(11), 1613–1615.
11. Ghosh, W., & Roy, P. (2006). *Mesorhizobium thiogangeticum* sp. nov., a novel sulfur-oxidizing chemolithoautotroph from rhizosphere soil of an Indian tropical leguminous plant. *International Journal of Systematic and Evolutionary Microbiology, 56*, 91–97.
12. Kumar, P. A., Jyothsna, T. S., Srinivas, T. N., Sasikala, C. H., Ramana, C. H. V., & Imhoff, J. F. (2007). *Marichromatium bheemlicum* sp. nov., a non-diazotrophic, photosynthetic gammaproteobacterium from a marine aquaculture pond. *International Journal of Systematic and Evolutionary Microbiology, 57*, 1261–1265.
13. Srinivas, T. N., Kumar, P. A., Sasikala, C. H., & Ramana, C. H. V. (2007). *Rhodovulum imhoffii* sp. nov. *International Journal of Systematic and Evolutionary Microbiology, 57*(2), 228–232.
14. Petri, R., Podgorsek, L., & Imhoff, F. (2001). Phylogeny and distribution of the *soxB* gene among thiosulfate-oxidizing bacteria. *FEMS Microbiology Letters, 197*, 171–178.
15. Meyer, B., Imhoff, J. F., & Kuever, J. (2007). Molecular analysis of the distribution and phylogeny of the *soxB* gene among sulfur-oxidizing bacteria - evolution of the Sox sulfur oxidation enzyme system. *Environmental Microbiology, 9*(12), 2957–2977.
16. Chen, L., Li, W., Liu, S., Tao, M., Long, Y., Duan, W., et al. (2009). Novel genetic markers derived from the DNA fragments of *sox* genes. *Molecular and Cellular Probes, 23*, 157–165.
17. Anandham, R., Indiragandhi, P., Madhaiyan, M., Ryu, K. Y., Jee, H. J., & Sa, T. M. (2008). Chemolithoautotrophic oxidation of thiosulfate and phylogenetic distribution of sulfur oxidation gene (*soxB*) in *rhizobacteria* isolated from crop plants. *Research in Microbiology, 159*, 579–589.
18. Anandham, R., Indiragandhi, P., Madhaiyan, M., Chung, J., Ryu, K. Y., Jee, H. J., et al. (2009). Thiosulfate oxidation and mixotrophic growth of *Methylobacterium goesingense* and *Methylobacterium fujisawaense*. *Journal of Microbiology and Biotechnology, 19*(1), 17–22.
19. Sakano, Y., & Kerkhof, L. (1998). Assessment of changes in microbial community structure during operation of an ammonia biofilter with molecular tools. *Applied and Environmental Microbiology, 64*, 4877–4882.
20. Kelly, D. P., Shergill, J. K., Lu, W. P., & Wood, W. P. (1997). Oxidative metabolism of inorganic sulfur compounds by bacteria. *Antonie Van Leeuwenhoek, 71*, 95–107.
21. Kelly, D. P., & Wood, A. P. (2000). Reclassification of some species of *Thiobacillus* to the newly designated genera *Acidithiobacillus* gen. nov., *Halothiobacillus* gen. nov. and *Thermithiobacillus* gen. nov. *International Journal of Systematic and Evolutionary Microbiology, 50*, 511–516.
22. Ghosh, W., Mallick, S., & Dasgupta, S. K. (2009). Origin of the Sox multienzyme complex system in ancient thermophilic bacteria and coevolution of its constituent proteins. *Research in Microbiology, 160* (6), 409–420.
23. Lahiri, C., Mandal, S., Ghosh, W., Dam, B., & Roy, P. (2006). A novel gene cluster soxSRT is essential for the chemolithotrophic oxidation of thiosulfate and tetrathionate by *Pseudaminobacter salicylatoxidans* KCT001. *Current Microbiology, 52*(4), 267–273.
24. Welte, C., Hafner, S., Krätzer, C., Quentmeier, A., Friedrich, C. G., & Dahl, C. (2009). Interaction between Sox proteins of two physiologically distinct bacteria and a new protein involved in thiosulfate oxidation. *FEBS Letters, 17*(583(8)), 1281–1286.
25. Wodara, C., Kostka, S., Egert, M., Kelly, D. P., & Friedrich, C. G. (1994). Identification and sequence analysis of the *soxB* gene essential for sulfur oxidation of *Paracoccus denitrificans* GB17. *Journal of Bacteriology, 176*(20), 6188–6191.
26. Lu, W.-P., & Kelly, D. P. (1983). Purification and some properties of two principal enzymes of the thiosulfate-oxidizing multi-enzyme system from *Thiobacillus* A2. *Journal of General Microbiology, 129*, 3549–3564.
27. Gonzalez, J. M., Kiene, R. P., & Moran, M. A. (1999). Transformation of sulfur compounds by an abundant lineage of marine bacteria in the subclass of the class *Proteobacteria*. *Applied and Environmental Microbiology, 65*, 3810–3819.
28. Swingley, W. D., Sadekar, S., Mastrian, S. D., Matthies, H. J., Hao, J., Ramos, H., et al. (2007). The complete genome sequence of *Roseobacter denitrificans r*eveals a mixotrophic rather than photosynthetic metabolism. *Journal of Bacteriology, 189*(3), 683–690.